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Minireview

How selfish retrotransposons are silenced in *Drosophila* germline and somatic cells

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Abstract Transposable elements (TEs) are DNA elements found in the genomes of various organisms. TEs have been highly conserved during evolution, suggesting that they confer advantageous effects to their hosts. However, due to their ability to transpose into virtually any locus, TEs have the ability to generate deleterious mutations in the host genome. In response, a variety of different mechanisms have evolved to mitigate their activities. A main defense mechanism is RNA silencing, which is a gene silencing mechanism triggered by small RNAs. In this review, we address RNA silencing mechanisms that silence retrotransposons, a subset of TEs, and discuss how germline and somatic cells are equipped with different retrotransposon silencing mechanisms.

Keywords: Argonaute; Retrotransposon; siRNA; piRNA; RNA silencing; *Drosophila*

1. RNA silencing involving PIWI proteins

Biochemical and mutation analyses have revealed the existence of Dicer and Argonaute multigene families, members of which are the key factors in RNA silencing in *Drosophila* [1–4]. Dicer functions in maturing small RNAs [20–30 nucleotides (nt)] that trigger RNA silencing, whereas Argonaute functions in down-regulating gene expression post-transcriptionally by directly targeting particular mRNAs [5]. mRNA targets are selected by base-pairing with small RNAs, which have been processed by Dicer and loaded onto the Argonautes. Extensive studies at the molecular level have revealed that two members of the *Drosophila* Argonaute family, Argonaute1 (AGO1) and Argonaute2 (AGO2), specifically associate with miRNA and siRNA, respectively, and function in gene silencing mechanisms mediated by miRNAs and RNAi, respectively [3]. We have analyzed gene silencing in living cells using monoclonal antibodies against each of the Argonaute proteins to immunopurify endogenous antigens and specifically associated small RNAs from living cells [6]. AGO1 and AGO2 have been well studied, therefore, we focused on identifying small RNA binding partners, as well as investigating RNA silencing pathways of other *Drosophila* Argonaute members, namely AGO3, Aubergine (Aub), and Piwi. These three Argonautes are most likely expressed only in the germline [7], and are collectively called the PIWI proteins to distinguish them from ubiquitously expressed AGO1 and AGO2 [8].

Forward genetic approaches have shown that mutations introduced into the *Piwi* gene cause disruption to the germline and that *Piwi* is an essential factor in germline stem cell (GSC) self-renewal in both males and females [9–11]. It was also demonstrated that *Piwi* mutations impact retrotransposon mobility [12,13]; without functional *Piwi*, retrotransposons become abnormally active. These were the first studies to show the connection between *Piwi* function and regulation of retrotransposon activity. However, the molecular function of Piwi protein had yet to be determined.

Genetic studies have shown that *Aub* is required for pole cell formation [14] and for activating RNAi during *Drosophila* oocyte maturation [15]. *Aub* is also involved in silencing retrotransposons in the germline [16–18], and in silencing *Stellate* genes in the testis by targeting the *Suppressor of Stellate* [*Su(Ste)*] repeats on the Y chromosome, which are highly homologous to *Stellate*.

As mentioned above, AGO1 and AGO2 specific monoclonal antibodies were key reagents for the molecular investigation of Argonaute functions in RNA silencing. Thus, we endeavored to produce monoclonal antibodies against the PIWI proteins and were indeed successful in producing specific monoclonal antibodies that recognize each of the antigens and that do not cross-react with other Argonaute members [19–21].

2. The PIWI proteins associate with rasiRNAs in the germline

Not only Piwi and Aub proteins but also AGO3 was successfully purified from *Drosophila* ovary lysates. Small RNAs contained in the immunoprecipitates from about 200 ovaries were visible by silver staining ([21] and unpublished data), indicating their abundance. Identification and analysis of these small RNAs revealed that all three PIWI proteins preferentially associate with a particular set of small RNAs, previously termed as rasiRNAs (repeat-associated small interfering RNAs) [22]. This indicates that the PIWI proteins most likely

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function in pathways silencing retrotransposons in the germline since rasiRNAs are mainly derived from retrotransposons, remnants of ancient retrotransposons or from other repetitive sequences found in the genome [22]. Particular characteristics of small RNAs associated with the PIWI proteins are that (1) Piwi and Aub show a strong preference to bind to rasiRNAs originating from the antisense transcripts of retrotransposons, whereas AGO3 prefers to bind rasiRNAs from the sense strand and (2) the preferred lengths of rasiRNAs that associate with each PIWI protein differ. Piwi prefers to associate with longer rasiRNAs (24–30 nt), while AGO3 prefers to bind to relatively smaller rasiRNAs of approximately 23 nt [20]. rasiRNAs are longer in length than miRNAs and siRNAs, which function in gene silencing in association with AGO1 and AGO2 [22]. Currently, small RNAs binding to the PIWI proteins in other organisms, including mouse and fish, are collectively referred to as piRNAs (PIWI-interacting RNAs) [23]; thus, we will follow this terminology.

3. piRNAs are methylated

In *Drosophila* piRNAs are chemically different from miRNAs [18,20,24,25]. As opposed to miRNAs expressed in plants, miRNAs in animals show sensitivity to peridate ($NaIO_4$) oxidation and beta-elimination treatments since they contain a 2′, 3′-*cis*-diol at their 3′ ends [26]. On the other hand, piRNAs are resistant to these chemical treatments, indicating that piRNAs lack one of the terminal hydroxyl groups [18,20,24,25]. Further investigation using the mutant fly, *piggyBac*^{f00810}, in which *CG12367* is disrupted by an element called *piggyBac*, revealed that piRNAs expressed in fly ovaries and those associated with the PIWI proteins are 2′-*O*-methylated at their 3′ ends [24,25]. The gene responsible for this modification is *DmHen1*/*Pimet* (piRNA methyltransferase) (dubbed *CG12367* in FlyBase), which is the *Drosophila* orthologue of the *A. thaliana* gene, *Hen1*, identified as a source of miRNA methyltransferase activity by Yu et al. [27]. Under conditions where the PIWI proteins physically associate with DmHen1/Pimet, the miRNA binding partner, AGO1, is not able to associate with the enzyme [24]. This might explain why miRNAs are not methylated in *Drosophila*. Phil Zamore and his colleagues showed that siRNAs exogenously introduced in S2 cells are also methylated [25]. Recently, we performed periodate oxidation and beta-elimination on siRNAs associated with AGO2 in S2 cell lysates that had been pre-incubated with siRNA duplexes, and we confirmed that exogenous siRNAs could indeed serve as substrates for DmHen1/Pimet (unpublished observations).

4. Association of AGO2 with endogenous siRNAs

Flies utilize the RNAi mechanisms to defend against viral infection [28]. siRNAs originating from infecting viruses that produce double-stranded RNAs (dsRNAs) as part of their replication cycle associate with AGO2 [28]. Mutations in *Ago2* and *Dicer2* caused loss of viral infection resistance, which strengthens the idea that the RNAi system is involved in virus defense.

A question we then raised was whether AGO2 could exist simply to lie in wait for exogenous small RNAs, such as siRNAs originating from the viral dsRNAs, or does AGO2 have its own endogenous partner(s) in naïve cells and organs. To address this question, we specifically immunoprecipitated endogenous AGO2 from naïve S2 cells that were grown under normal conditions, and visualized small RNAs associated with the protein [29]. We found that in these cells AGO2 existed in association with endogenous small RNAs of around 21 nt. Interestingly, the size peak was 1 nt shorter than that of miRNAs associated with AGO1 in S2 cells. Since this was the first demonstration that AGO2 has its own endogenous small RNA partners, as do other Argonautes in *Drosophila*, we referred to them as endogenous short interfering RNA, esiRNA. Identification and analysis of esiRNAs revealed that they are mainly derived from retrotransposons and other genomic repetitive elements. This property of esiRNAs resembled that of piRNAs. However, piRNAs and esiRNAs are clearly distinct classes of small RNAs in respect to their sizes and their protein partners, as piRNAs bind to the PIWI proteins and their sizes are about 24–30 nt in length. In contrast, esiRNAs are approximately 21 nt and associate specifically with AGO2. The expression profiles of piRNAs and esiRNAs through development are also different; piRNAs are found in principle only in the germline, while esiRNAs are likely to be expressed ubiquitously, based on the observation that esiRNAs could be detected, not only in S2 cells, but also in adult bodies devoid of germline, as well as in embryos (unpublished data) where most of the cells are somatic.

5. piRNA and esiRNA biogenesis

piRNAs associated with Piwi and Aub in ovaries, show a strong preference for uracil (U) at the 5′ ends, while AGO3-associated piRNAs show a strong preference for adenosine (A) at the 10th nucleotide from the 5′ ends. By contrast, esiRNAs show little or no bias for nucleotides at any position, indicating that the processing mechanisms of piRNAs and esiRNAs might be different. In early 2007, the Hannon group and ourselves proposed a model for "piRNA biogenesis" [20,30]. In agreement with the observation of Vagin et al. [18], in which piRNAs were produced Dicer-independently, our model also excludes Dicer activities. Although it is not yet entirely understood, it is generally accepted that the Slicer activities of PIWI proteins [19–21] contribute to the generation of piRNAs, at least in determining and producing their 5′ ends.

How, then, are esiRNAs produced in vivo? Using bioinformatic analyses of esiRNAs, we summed the number of unique small RNAs in a 5 kb sliding window and plotted this against the *Drosophila* draft genome assembly. Clusters of small RNA production were observed, from which we estimated that most esiRNAs could be divided into two types: those that matched retrotransposons and those that arose from long stem–loop structures from repetitive sequences located on the X chromosome. Plotting of the esiRNAs derived from retrotransposons against the *Drosophila* draft genome assembly revealed a distinct "hotspot" that produces a number of esiRNAs and that the hotspot expresses esiRNAs from both sense and antisense strands. By looking at the plotting pattern, we postulated that esiRNAs must be largely derived from dsRNAs arising from the bi-directional transcripts of retrotransposons in a Dicer2-dependent manner. By contrast, esiRNAs arising from stem–

loop structures showed a strong strand bias, indicating that their production must be Dicer2-independent. However, it was subsequently found that in the latter case the precursor presumably folds into a long stem–loop structure, and the esiRNAs must arise from the long stem region. This further supported the idea that esiRNAs are Dicer2-dependently produced.

We then assessed whether or not the production and normal accumulation of esiRNAs require Dicer2. We depleted Dicer2 and other small RNA processing factors from S2 cells one by one by RNAi and monitored the abundance of esiRNAs. A marked reduction of esiRNAs in Dicer2-depleted S2 cells was observed, whereas Drosha and Dicer1 depletion did not affect esiRNA accumulation. esiRNAs were hardly detectable in Dicer2 mutant ovaries. There results indicated that esiRNAs are produced in a Dicer2-dependent manner.

As mentioned above, exogenous siRNAs processed from long dsRNAs by Dicer2 and loaded onto AGO2 resisted periodate oxidation and beta-elimination reactions because they are methylated at their 3′ ends. We, therefore, wondered if esiRNAs, associated with AGO2 in S2 cells, are also methylated in vivo. To address this, esiRNAs, associated with AGO2 in S2 cells, were subjected to periodate oxidation and beta-elimination. esiRNAs in *DmHen1*/*Pimet* mutant ovaries were also subjected to these reactions. The results clearly indicated that esiRNAs are also methylated. Furthermore, the gene responsible for the esiRNA modification was *DmHen1*/*Pimet*. These data strongly support the idea that esiRNAs are processed by Dicer2, in the same manner as siRNAs originating from exogenous dsRNAs.

6. Specific association of esiRNAs with AGO2

AGO2 in *Drosophila* is known to exhibit Slicer activity [6]. In our recent study, we showed that the AGO2-esiRNA complexes, immunopurified from S2 cells, had activity for cleaving an RNA target (esiRNA-sl-1 target) harboring a sequence completely matching one found in the most abundant esiRNA (esiRNA-sl-1) in the complexes. However, another target with a sequence complementary to *bantam*miRNA (bantam target), one of miRNAs expressed in S2 cells, was not cleaved by the complexes, indicting that at least bantam is not loaded onto AGO2.*bantam* was previously shown to be associated with AGO1 in S2 cells and AGO1 complexes immunopurified from S2 cells were able to cleave the *bantam* target [3], indicating that AGO1 also has Slicer activity, depending on the sequence of the small RNA with which AGO1 is associated. Recently, we found that the AGO1 complexes isolated from S2 cells were not able to cleave the esiRNA-sl-1 target, suggesting that esiRNAs are not loaded onto AGO1 in vivo.

One might then ask if all *Drosophila* miRNAs are exclusively loaded onto AGO1, or are some sorted onto AGO2 as has been suggested by the Forstemann et al. [31]. We characterized miRNAs in an AGO2-associated small RNA library. It was found that, for example, miR-20071, which was one of the newly identified miRNAs in the study, did indeed bind to AGO2, as assayed by northern blotting. However, the association was not exclusive to AGO2. Rather, it was more strongly associated with AGO1. Respective sorting of miRNAs and siRNAs into association with AGO1 and AGO2 was again strongly supported.

7. Retrotransposon silencing by the AGO2–esiRNA complex

It has been previously shown that loss of PIWI proteins in the germline caused abnormally high expression of retrotransposon transcripts. Does loss of esiRNAs, or rather, loss of the AGO2–esiRNA-mediated silencing pathway, cause the same phenomenon in somatic cells? To address this question, we have performed qRT-PCR to detect expression of retrotransposons from total RNAs isolated from S2 cells, as well as from adult male flies devoid of testes. It was clearly indicated that loss of Dicer2, the esiRNA processing factor, caused higher expression of *297*, *1731* and other retrotransposons but not of *rp49* (also known as *RpL32*), a ribosomal protein gene used as a control, which suggests that somatic expression of retrotransposons is suppressed by the AGO2–esiRNA pathway. All these results in somatic cells, together with previous data from the germline [18], indicate that retrotransposon silencing occurs in both these *Drosophila* cell types but that they utilize different molecules/pathways for silencing the selfish elements. Since the Piwi proteins and piRNAs are not detectable in S2 cells, at least in our laboratory, we assume that the retrotransposon silencing pathway mediated by piRNAs and the PIWI proteins does not function in S2 cells. But, retrotransposons are, to some extent, active even in somatic cells, potentially causing lethal mutations. For this reason, somatic cells may have evolved a pathway for silencing transposable genes (Fig. 1). Subsequently, *Drosophila* somatic cells may have modified this pathway to confer protection from invasive viruses that produce dsRNAs during the replication cycle.

We wonder if somatic cells in ovaries and testes use the AGO2–esiRNA pathway, as in S2 cells. AGO2 is expressed throughout ovary development, but in early-stage oocytes RNAi seems to be inactive [15]. Thus, even though somatic cells in the germline are equipped with the AGO2–esiRNA pathways, they may not be active. In the germline, retrotransposons are thought to be very active and thus there is a large chance for the genome to be invaded by the mobile genes. To efficiently repress this deleterious activity, the germline may have acquired the additional system(s) mediated by the PIWI proteins and piRNAs (Fig. 1).

We experimentally showed that the AGO2–esiRNA complexes exhibit Slicer activity. This suggests that the complexes might silence retrotransposon genes by transcript cleavage. However, studies in the fission yeast *Schizosaccharomyces pombe* have shown that RNAi contributes to the formation of heterochromatin [32]. Similarly, a loss of *Dicer2* or *Ago2* activity is correlated with defects in heterochromatin formation in *Drosophila* [33,34]. Thus, we propose that the AGO2–esiRNA complex induces heterochromatin formation at specific chromosomal regions containing a high density of retrotransposon genes.

7.1. Additional proteins of the AGO2–esiRNA pathway

It should be noted that we are not the sole group to have identified esiRNAs in *Drosophila* somatic cells [35–37]. Three other groups in the United States also reported identification and characterization of *Drosophila* somatic esiRNAs (in their studies, the small RNAs are referred to as endo-siRNAs). In agreement with us, Ghildiyal et al. reported that endo-siRNAs show high homology to retrotransposons [35]. It was also concluded that endo-siRNAs are produced by Dicer2, although

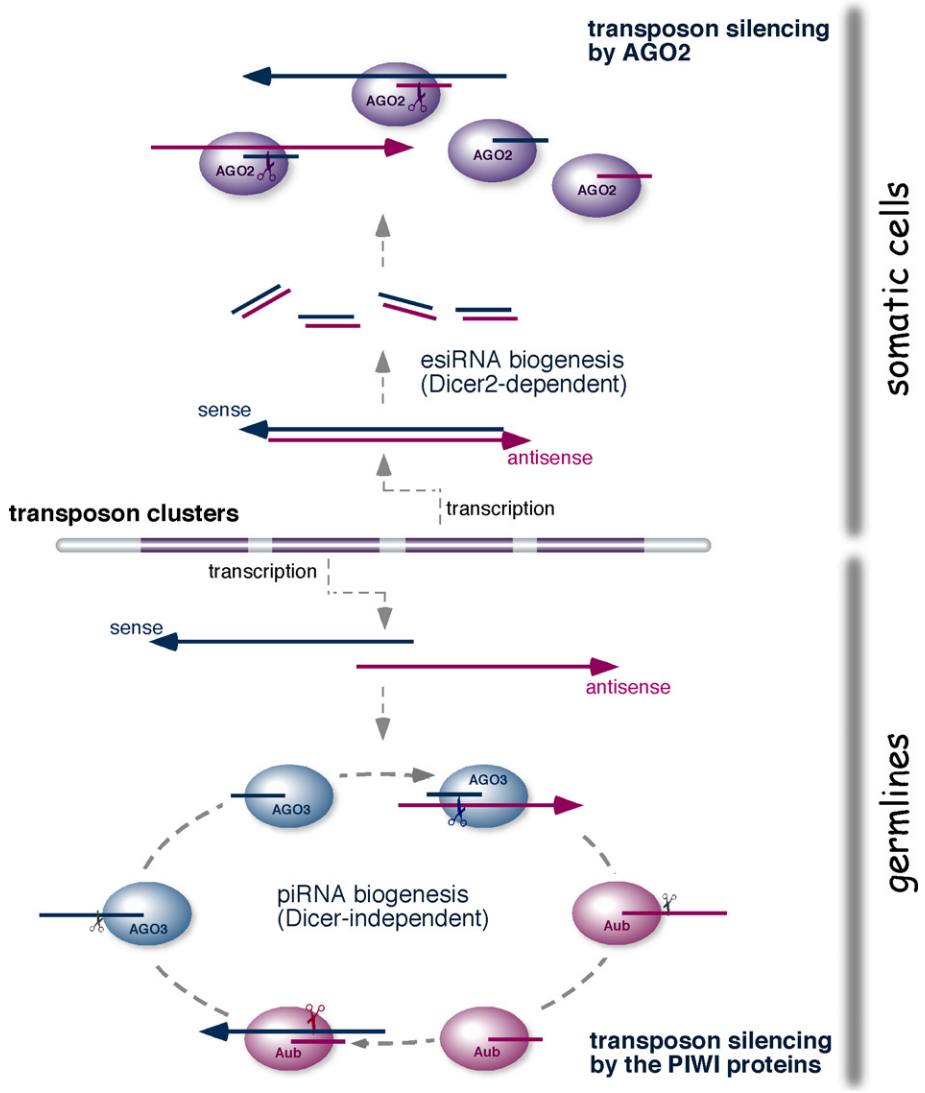

Fig. 1. Predicted actions of retrotransposon silencing in *Drosophila* germline and somatic cells. Although the extent may differ, retrotransposons are active and transposable in both germline and somatic cells. In the germline, Aub, Piwi, and AGO3, the germline-specific Argonautes, function in silencing selfish DNA elements by associating with piRNAs that originate from retrotransposon transcripts from both sense and antisense directions. It has previously been demonstrated that piRNA biogenesis occurs Dicer-independently and the Slicer activities of the germline-specific Argonautes are involved in the process. By contrast, in somatic cells, where Aub, Piwi, AGO3, and piRNAs are hardly detectable, AGO2 and esiRNAs function in an equivalent manner. It should be noted that esiRNAs are processed in a Dicer2-dependent manner, unlike piRNAs. It is speculated that *Drosophila* has evolved two distinct machineries/pathways to make selfish DNA elements silent, one for the germline and the other for somatic cells. The AGO2–esiRNA complexes may also function in the germline. In the germline, retrotransposons might be so active that additional mechanisms are required to effectively silence these selfish genes.

some endo-siRNAs were shown to persist in *dicer2* mutants. One of the peculiar observations made by both Czech et al. and Okamura et al. is that *Loquacious*, a factor known to be involved in miRNA processing, along with *Dicer1* [38–40], is also required for endo-siRNA processing, along with Dicer2 [36,37]. An additional observation made by both groups is that some endo-siRNAs target transcripts of regular protein-coding genes. It is most likely that Ago2–endo-siRNA complexes are not solely dedicated to silencing retrotransposons and other repetitive genes.

Our findings, together with related studies, potentially have an important implication in human disease; AGO2 is known to form a complex with the *Drosophila* homolog of fragile X mental retardation protein (FMRP) [41,42]. This provides a link between transposon and protein-coding gene silencing by AGO2 and post-transcriptional gene control by FMRP [43].

7.2. Are similar mechanisms found in other species?

Two independent groups simultaneously reported the identification and characterization of endogenous siRNAs (endo-siRNAs) expressed in mouse oocytes [44,45]. Unlike mouse piRNAs, endo-siRNAs are about 21 nt and originate from many different retrotransposons in a Dicer-dependent manner. Loss of *Dicer* activity caused higher expression of particular retrotransposons, such as RLTR10 and IAP, in mouse ovaries. By these criteria, we understand that mouse endo-siRNAs are equivalent to or are involved in the same group as *Drosophila* esiRNAs. However, which mouse Argonautes, PIWI proteins

or non-PIWI proteins, the endo-siRNAs are associated with is still unknown. Raising antibodies against each mouse Argonaute protein (mouse possesses eight Argonaute genes) would provide very helpful reagents to address these questions.

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References

[1] Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R. and Hannon, G.J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293, 1146–1150.
[2] Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363–366.
[3] Okamura, K., Ishizuka, A., Siomi, H. and Siomi, M.C. (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. 18, 1655–1666.
[4] Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J. and Carthew, R.W. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117, 69–78.
[5] Hammond, S.M. (2005) Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. 579, 5822–5829.
[6] Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. and Siomi, M.C. (2005) Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. Genes Dev. 19, 2837–2848.
[7] Williams, R.W. and Rubin, G.M. (2002) ARGONAUTE1 is required for efficient RNA interference in *Drosophila* embryos. Proc. Natl. Acad. Sci. USA 99, 6889–6894.
[8] Peters, L. and Meister, G. (2007) Argonaute Proteins: mediators of RNA silencing. Mol. Cell 26, 611–623.
[9] Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D. and Lin, H. (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. 12, 3715–3727.
[10] Cox, D.N., Chao, A. and Lin, H. (2000) piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development 127, 503–514.
[11] Szakmary, A., Cox, D.N., Wang, Z. and Lin, H. (2005) Regulatory relationship among piwi, pumilio, and bag-of-marbles in *Drosophila* germline stem cell self-renewal and differentiation. Curr. Biol. 15, 171–178.
[12] Sarot, E., Payen-Groschene, G., Bucheton, A. and Pelisson, A. (2004) Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster* flamenco gene. Genetics 166, 1313–1321.
[13] Kalmykova, A.I., Klenov, M.S. and Gvozdev, V.A. (2005) Argonaute protein PIWI controls mobilization of retrotransposons in the *Drosophila* male germline. Nucleic Acids Res. 33, 2052–2059.
[14] Harris, A.N. and Macdonald, P.M. (2001) Aubergine encodes a *Drosophila* polar granule component required for pole cell formation and related to eIF2C. Development 128, 2823–2832.
[15] Kennerdell, J.R., Yamaguchi, S. and Carthew, R.W. (2002) RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on aubergine and spindle-E. Genes Dev. 16, 1884–1889.
[16] Vagin, V.V., Klenov, M.S., Kalmykova, A.I., Stolyarenko, A.D., Kotelnikov, R.N. and Gvozdev, V.A. (2004) The RNA Interference proteins and Vasa locus are involved in the silencing of retrotransposons in the female germline of *Drosophila melanogaster*. RNA Biol. 1, 54–58.
[17] Savitsky, M., Kwon, D., Georgiev, P., Kalmykova, A. and Gvozdev, V. (2006) Telomere elongation is under the control of the RNAi-based mechanism in the *Drosophila* germline. Genes Dev. 20, 345–354.
[18] Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. and Zamore, P.D. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. Science 313, 320–324.
[19] Saito, K., Nishida, K.M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. and Siomi, M.C. (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. Genes Dev. 20, 2214–2222.
[20] Gunawardane, L.S., Saito, K., Nishida, K.M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H. and Siomi, M.C. (2007) A Slicer-mediated mechanism for repeat-associated siRNA 5′ end formation in *Drosophila*. Science 315, 1587–1590.
[21] Nishida, K.M., Saito, K., Mori, T., Kawamura, Y., Nagami-Okada, T., Inagaki, S., Siomi, H. and Siomi, M.C. (2007) Gene silencing mechanisms mediated by Aubergine piRNA complexes in *Drosophila* male gonad. RNA 13, 1911–1922.
[22] Aravin, A.A. et al. (2003) The small RNA profile during *Drosophila melanogaster* development. Dev. Cell 5, 337–350.
[23] O'Donnell, K.A. and Boeke, J.D. (2007) Mighty Piwis defend the germline against genome intruders. Cell 129, 37–44.
[24] Saito, K., Sakaguchi, Y., Suzuki, T., Suzuki, T., Siomi, H. and Siomi, M.C. (2007) Pimet, the *Drosophila* homolog of HEN1, mediates 2′-*O*-methylation of Piwi-interacting RNAs at their 3′ ends. Genes Dev. 21, 1603–1608.
[25] Horwich, M.D., Li, C., Matranga, C., Vagin, V., Farley, G., Wang, P. and Zamore, P.D. (2007) The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. Curr. Biol. 17, 1265–1272.
[26] Hutva'gner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T. and Zamore, P.D. (2001) Science 293, 834–838.
[27] Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R.W., Steward, R. and Chen, X. (2005) Methylation as a crucial step in plant microRNA biogenesis. Science 307, 932–935.
[28] Li, H., Li, W.X. and Ding, S.W. (2002) Induction and suppression of RNA silencing by an animal virus. Science 296, 1319–1321.
[29] Kawamura, Y. et al. (2008) *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature, doi:10.1038/nature06938.
[30] Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R. and Hannon, G.J. (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell 128, 1089–1103.
[31] Förstemann, K., Horwich, M.D., Wee, L., Tomari, Y. and Zamore, P.D. (2007) *Drosophila* microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. Cell 130, 287–297.
[32] Bühler, M. and Moazed, D. (2007) Transcription and RNAi in heterochromatic gene silencing. Nat. Struct. Mol. Biol. 14, 1041–1048.
[33] Peng, J.C. and Karpen, G.H. (2007) H3K9 methylation and RNA interference regulate nucleolar organization and repeated DNA stability. Nature Cell. Biol. 9, 25–35.
[34] Deshpande, G., Calhoun, G. and Schedl, P. (2005) *Drosophila* argonaute-2 is required early in embryogenesis for the assembly of centric/centromeric heterochromatin, nuclear division, nuclear migration, and germ-cell formation. Genes Dev. 19, 1680–1685.
[35] Ghildiyal, M. et al. (2008) Endogenous siRNAs derived from transposons and mRNAs in *Drosophila* somatic cells. Science, doi:10.1126/science.1157396.
[36] Czech, B. et al. (2008) An endogenous small interfering RNA pathway in *Drosophila*. Nature, doi:10.1038/nature07007.
[37] Okamura, K., Chung, W., Ruby, J.G., Guo, H., Bartel, D.P. and Lai, E.C. (2008) The *Drosophila* hairpin RNA pathway generates endogenous short interfering RNAs. Nature, doi:10.1038/nature07015.
[38] Forstemann, K. et al. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol. 3, e236.
[39] Jiang, F., Ye, X., Liu, X., Fincher, L., McKearin, D. and Liu, Q. (2005) Dicer-1 and R3D1-L catalyze microRNA maturation in *Drosophila*. Genes Dev. 19, 1674–1679.

[40] Saito, K., Ishizuka, A., Siomi, H. and Siomi, M.C. (2005) Processing of pre-microRNAs by the Dicer-1–Loquacious complex in *Drosophila* cells. PLoS Biol. 3, e235.
[41] Caudy, A.A., Myers, M., Hannon, G.J. and Hammond, S.M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev. 16, 2491–2496.
[42] Ishizuka, A., Siomi, M.C. and Siomi, H. (2002) A *Drosophila* protein interacts with components of RNAi and ribosomal proteins. Genes Dev. 16, 2497–2508.
[43] Laggerbauer, B. et al. (2001) Evidence that fragile X mental retardation protein is a negative regulator of translation. Hum. Mol. Genet. 10, 329–338.
[44] Tam, O.H. et al. (2008) Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. Nature 1, doi:10.1038/nature06904.
[45] Watanabe, T. et al. (2008) Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature, doi:10.1038/nature06908.